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REPORT NO. 861

THERMAL INACTIVATION OF *E. COLI* AND
ITS INTRACELLULAR β -GALACTOSIDASE

(Interim Report)

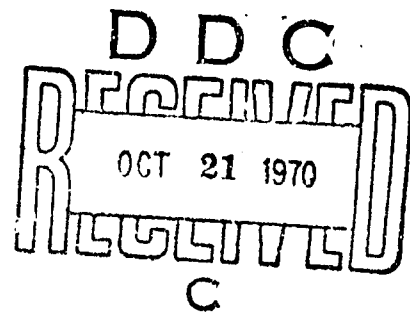
by

Dorothy M. Witt, M.S.

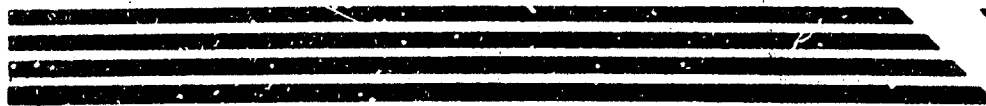
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Arnold S. Brownell, Ph.D.

17 March 1970



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Fort Knox, Kentucky 40121

17 March 1970

Models and Mechanisms of the Effects of Laser Radiation
on Biological Systems.

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USAMRL REPORT NO. 861
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ABSTRACT

THERMAL INACTIVATION OF *E. COLI* AND ITS INTRACELLULAR β -GALACTOSIDASE

OBJECTIVE

To determine whether the kinetics of thermal inactivation of *E. coli* K12/37 and its intracellular enzyme, β -galactosidase, are suitable for inclusion in systems used to evaluate mathematical models for analyzing thermal effects of laser radiation.

RESULTS

Reaction rate constants for the two systems have been determined, and the inactivation rates appear to be approximately first order within the limited temperature range studied.

CONCLUSIONS

Since the kinetics of the two thermal inactivation systems investigated are relatively simple, these systems can be incorporated into a system for evaluating models which predict mathematically the thermal damage resulting from laser radiation.

THERMAL INACTIVATION OF *E. COLI* AND ITS INTRACELLULAR β -GALACTOSIDASE

INTRODUCTION

The biological effect of an input of thermal energy, from a radiation source such as a laser, is caused by a rise in temperature that continuously varies with time. The temporal (time) integral of the rate of damage depending upon temperature should be indicative of the total damage. Attempts to sum the damage rates as a function of temperature change for complex systems such as skin have met with limited success because of difficulties in either measuring the temperature of the skin during the thermal episode or calculating the temperature of the skin since its thermal properties are uncertain.

If a simple system could be developed of which the time-temperature history and biological sensitivity could be determined under conditions approximating those in which laser injury occurs, this system could be used to evaluate mathematical models for analyzing thermal effects in more complex situations. Such a system must meet two requirements. One is that accurate measurements can be made of the spatial and temporal changes in temperature. The other is that quantitative measurements of thermal injury can be readily determined.

This study is an investigation of the kinetics of thermal inactivation of the bacterium *E. coli* and one of its intracellular enzymes, β -galactosidase, to determine whether these reaction kinetics are simple enough to make them useful in the analysis of thermal effects resulting from laser radiation. If the inactivation rates are essentially first order, the reaction constants can be utilized in relatively simple mathematical expressions for testing the usefulness of specific predictive models for subsequent use in more complex systems such as skin.

METHODS

A large culture of *E. coli* K12/37, a cryptic mutant isolated in this laboratory from the wild type, was grown in a synthetic medium (Spizizen's minimal medium (1)) with 0.5% glycerol as the carbon source and 5×10^{-4} M isopropyl- β -D-thiogalactopyranoside (IPTG) as the inducer of β -galactosidase synthesis. When the cells were fully induced and had begun the stationary phase of growth, they were harvested by centrifugation in the cold, washed with Spizizen's minimal medium to remove the IPTG, thus preventing further induction, and then resuspended in the minimal medium containing 5% by volume of glycerol. Aliquots were then frozen in a dry ice-alcohol bath and stored at -70°C . The rapid freezing in a glycerol medium and the low temperature storage maintain essentially 100% viability of the cells and protect the

¹Spizizen, J. Transformation of biochemically deficient strains of *Bacillus subtilis* by deoxyribonuclease. Proc. Nat. Acad. Sci. 44: 1072, 1958.

β -galactosidase from any loss of activity. The same batch of frozen stock was used for all experiments to ensure uniformity of enzyme content and to eliminate the effects of possible variation in heat sensitivity from one batch of cells to another.

Prior to an experiment, the frozen stock was rapidly thawed, diluted to the desired concentration with Spizizen's minimal medium, and the resulting cell suspension held in an ice bath.

Inactivations were carried out in a glass chamber surrounded with a jacket through which water of the desired temperature circulated. Fifteen ml of Spizizen's minimal medium were introduced into the chamber and stirred with a magnetic stirring bar. Temperature in the chamber was monitored continuously with a copper-constantan thermocouple and recording potentiometer. The temperature could be measured within 0.1°C . When the temperature within the chamber had equilibrated, 0.1 ml of cells was diluted into the medium using an Eppendorf pipette with a warmed tip. This procedure resulted in a drop in temperature of about 0.2°C , the original temperature being regained within a half minute. Because of this initial drop in temperature, sampling was not begun until the temperature had returned to the desired level. At appropriate intervals, 0.1 ml samples were withdrawn from the chamber using an Eppendorf pipette. Since the pipette tips were warmed to the same temperature as the cell suspensions, no detectable drop in temperature in the chamber occurred.

When cell inactivation experiments were being conducted, the samples were immediately diluted into 10 ml of chilled minimal medium to halt the inactivation process. After further appropriate dilutions, pour plates were made with nutrient agar. Plates were counted after 48 hours of incubation at 37°C . Control cell levels were determined by diluting 0.1 ml of chilled cells into 15 ml of Spizizen's minimal medium at room temperature and treating 0.1 ml of the diluted suspension in the same manner as the samples from the inactivation chamber.

For enzyme inactivation experiments, the 0.1 ml samples were diluted into 0.04 ml of toluene and 5.0 ml of chilled Spizizen's minimal medium, containing in each ml 50 μg chloramphenicol, 100 μg bovine serum albumin, and 12.5 μg sodium deoxycholate, and then mixed vigorously on a vortex mixer. The toluene and deoxycholate served to break down the cells and release the enzyme and the chloramphenicol to inhibit bacterial growth. The purpose of the albumin was to protect the enzyme since it tends to lose its activity in dilute solutions. After evaporation of the toluene in a water bath at 37°C , the crude extracts were assayed for β -galactosidase as previously described (2). Control enzyme levels were determined in the same way on a 0.1 ml sample of diluted cells.

²Brownell, A. S. and Dorothy M. Witt. Ultraviolet light inhibition of galactoside permease induction. *Biochem. Biophys. Res. Commun.* 21: 113, 1965; USAMRII Report No. 648, 1965 (DRC AD No. 627339).

RESULTS AND DISCUSSION

Thermal inactivation of β -galactosidase. Figure 1 presents a typical inactivation curve for each of the temperatures employed and illustrates that the kinetics of inactivation approximate those of a first order reaction within the temperature range of 58.9°C to 63.5°C. Although control enzyme levels were determined for each experiment, they were not plotted on the graph as zero time values because the initial drop in temperature in the chamber made them invalid as 100% values.

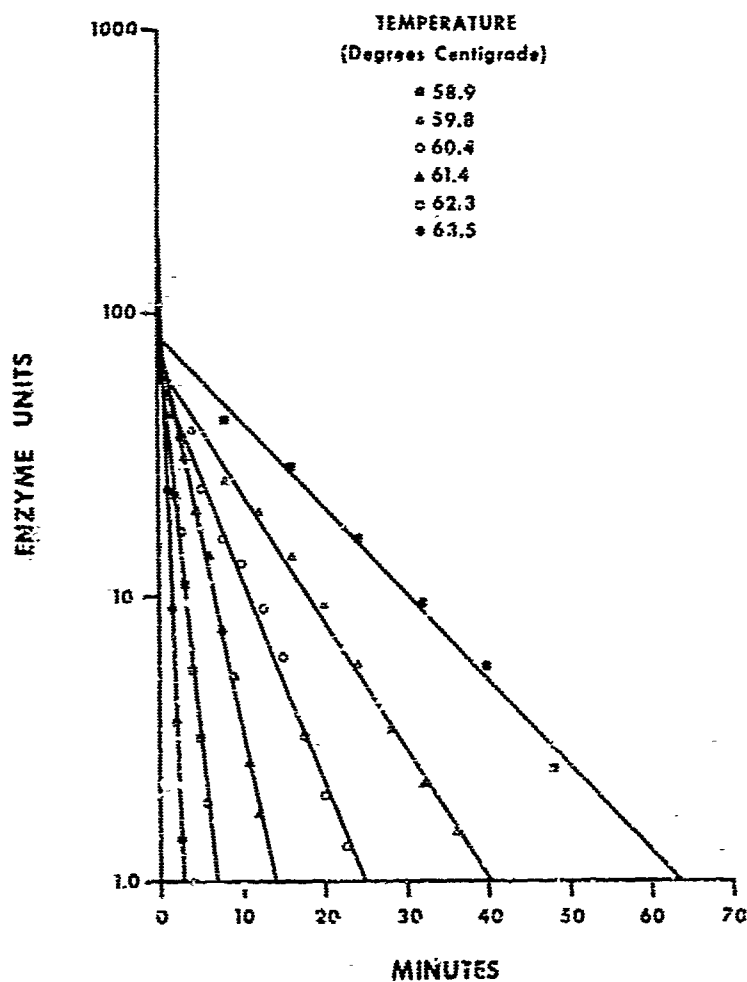


Fig. 1. Thermal inactivation of intracellular β -galactosidase at temperatures ranging from 58.9°C to 63.5°C. The logarithm of the enzyme activity is plotted as a function of time of exposure for each temperature.

The inactivation constant in sec^{-1} for each temperature was calculated from the slope of the line as determined by a least squares fit of the data. These inactivation constants, together with the mean and standard deviation for each temperature, are listed in Table 1. There was good agreement in the constants calculated for each temperature except for the first value determined at 58.9°C . There was also a good deal more scatter in the points on the inactivation curves for this temperature. The calculated standard deviation was 20% for the inactivation constant at 58.9°C while in the other cases it ranged from 7 to 16%.

TABLE 1
Reaction Rate Constants for Thermal Inactivation
of Intracellular β -Galactosidase

Temperature ($^\circ\text{C}$)	$k' \times 10^3$					
	58.9	59.8	60.4	61.4	62.5	63.5
	1.50	1.72	2.53	6.77	11.4	30.5
	0.94	1.84	3.22	5.13	11.4	23.0
	1.01	1.59	3.19	5.42	11.7	32.4
	1.02	1.86	2.53	6.15	13.6	33.9
	1.11	1.76	2.84	5.92	11.7	31.5
		1.83	2	5.16	10.9	29.2
		1.96	2	4.01	10.8	30.7
		1.67	2.4	4.64	10.6	30.8
						26.7
						26.9
Mean, standard deviation	1.12 ± 0.22	1.78 ± 0.12	2.70 ± 0.35	5.40 ± 0.87	11.5 ± 0.9	30.1 ± 2.4
$t_{1/10}^*$	35.6 ± 5.6	21.2 ± 1.3	14.4 ± 1.8	7.29 ± 1.2	3.36 ± 0.26	2.27 ± 0.11

*Time in minutes for 90% of the enzyme to be inactivated.

The $t_{1/10}$ value for each temperature is also listed in Table 1. This figure is the exposure time in minutes when 10% of the enzyme still remains in an active form.

When the logarithm of the inactivation constant was plotted against the reciprocal of the absolute temperature, a linear relationship resulted (Fig. 2). Each point on the curve is an average of at least five determinations of the inactivation constant at that particular temperature.

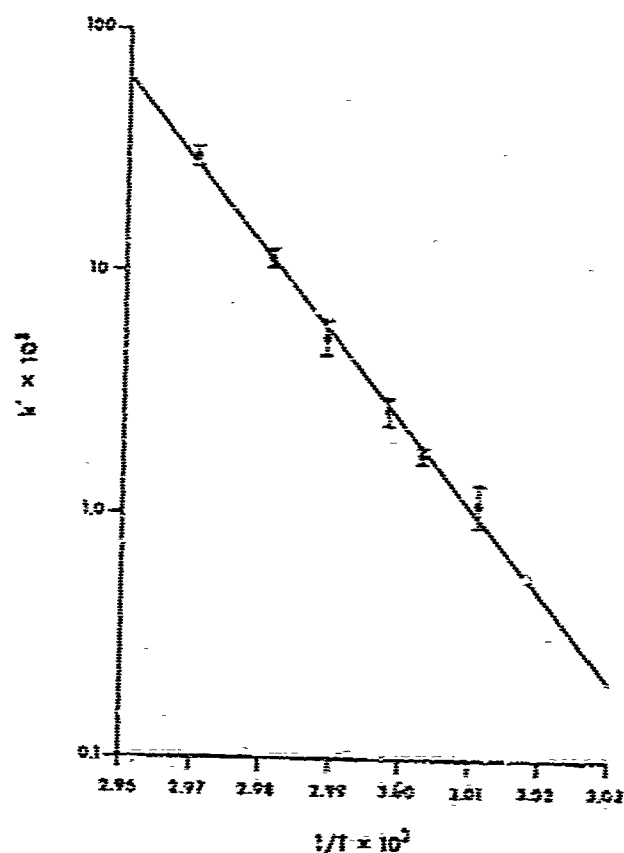


Fig. 2. Plot of the logarithm of the inactivation constant of intracellular β -galactosidase as a function of the reciprocal of the absolute temperature.

From this curve, it is obvious that the inactivation rate constant is temperature dependent and that the curve can be approximately represented by the equation,

$$\frac{d \ln k'}{dT} = -\frac{AE^*}{RT^2} \quad (1)$$

where k' is the rate constant, T the absolute temperature, R the gas constant in calories, and AE^* a quantity characteristic of the reaction with the dimensions of energy.

Integrating and assuming AE^* to be a constant,

$$\ln k' = -\frac{AE^*}{RT} + p'$$

or

$$k' = P e^{-\Delta E^*/RT} \quad (3)$$

where P is a constant of integration. ΔE^* can be readily determined from the slope of the line and turns out to be 152 kcal/M.

The other thermodynamic parameters can be calculated. The free energy of inactivation, ΔF^* , can be determined using Eyring's equation based on the theory of absolute reaction rates

$$k = \frac{kT}{h} e^{-\Delta F^*/RT} \quad (4)$$

where R is the gas constant in cal/mole/degree, N is Avogadro's number, h is Planck's constant, T is the absolute temperature, and k is the rate constant for that particular temperature. Within the temperature limits of this study, ΔF^* ranged from 24.0 to 22.1 kcal/M which is within the range found for the denaturation of other proteins (5).

It must be pointed out that these values of ΔE^* and ΔF^* are valid only if the reaction meets the necessary thermodynamic requirements. Since the system employed in these experiments is not a single molecular species, it is possible that the inactivation of β -galactosidase may be complicated by the presence of other cellular components. However, the reaction appears to satisfy equation (1) within the temperature limits of these experiments. Whether the relationship is the same at the higher temperatures reached during typical brief exposures to laser radiation remains to be determined.

Thermal inactivation of *E. coli* K12/37. The thermal killing of *E. coli* K12/37 cells appears to follow first order reaction rates also. Representative survival curves following exposures to temperatures between 51.0°C and 55.6°C are shown in Figure 3. As was the case with the thermal inactivation of β -galactosidase, there seems to be more scatter in the points on the curves at the lower temperatures. A minimum of six experiments was done at each temperature, and the inactivation constants in sec^{-1} determined from the slopes of the lines. These constants and the mean and standard deviation for each temperature are compiled in Table 2. The calculated standard deviation from the mean was found to range from 9 to 10%.

A plot of the logarithm of the inactivation constant against the reciprocal of the absolute temperature (Fig. 4, page 8) demonstrates that the rate constant is temperature dependent and can also be represented by equation (1). ΔE^* calculated from the slope of the line is 150 kcal/M. ΔF^* calculated from equation (4) ranges from 23.5 to 21.7 kcal/M within the temperature range used in these experiments.

⁵Joly, M. A Physico-chemical Approach to the Denaturation of Proteins. London and New York: Academic Press, 1965.

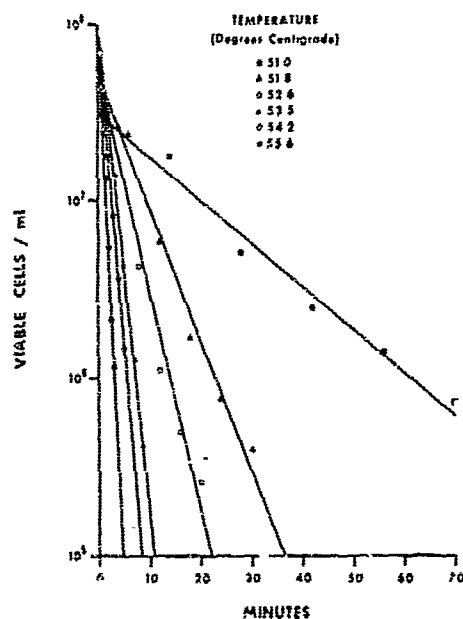


Fig. 3. Thermal inactivation of *E. coli* K12/37 at temperatures ranging from 51.0°C to 55.6°C. The logarithm of the number of viable cells is plotted as a function of time of exposure for each temperature.

TABLE 2
Reaction Rate Constants for Thermal Inactivation of *E. coli* K12/37

Temperature (°C)	$k' \times 10^3$					
	51.0	51.8	52.6	53.5	54.2	55.6
	0.385	1.69	4.05	8.11	16.9	27.6
	1.25	1.93	3.46	8.60	16.0	25.8
	0.917	2.83	2.99	8.52	10.4	25.2
	0.923	2.60	3.55	9.38	17.2	28.3
	1.20	2.54	4.82	9.16	12.7	24.7
	1.11	2.84	4.74	9.20	15.9	31.8
		2.27	4.09	11.2	15.4	
				13.8	19.0	
Mean, standard deviation	1.05±0.16	2.39±0.44	3.96±0.67	9.75±1.88	15.4 ±2.7	27.2 ±2.6
$t_{1/10}^*$	37.5 ±5.5	16.7 ±3.6	10.0 ±1.8	4.05±0.64	2.56±0.54	1.43±0.14

*Time in minutes for 90% of the cells to be inactivated.

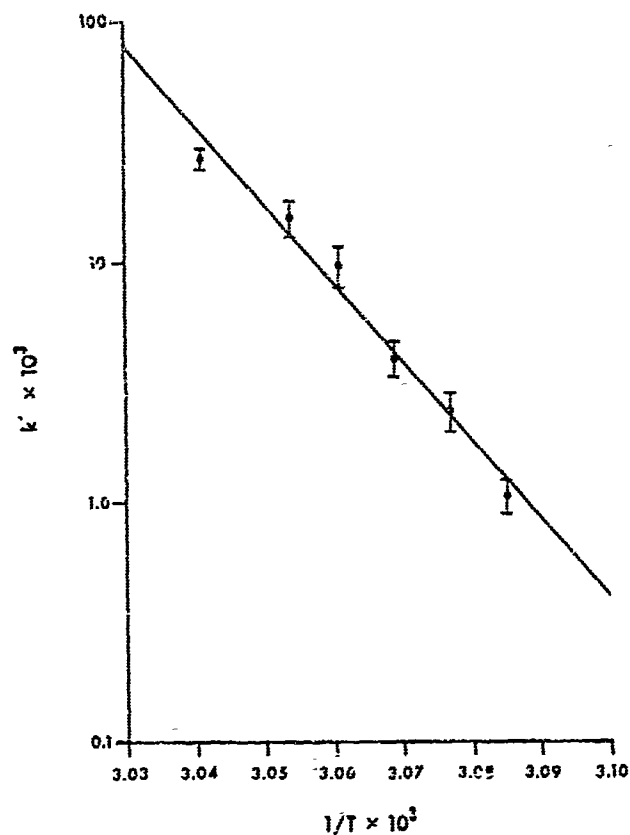


Fig. 4. Plot of the logarithm of the inactivation constant of *E. coli* K12/37 as a function of the reciprocal of the absolute temperature.

CONCLUSIONS

The kinetics of the thermal inactivation of *E. coli* K12/37 and the enzyme, β -galactosidase, do not appear to be complex in the temperature range studied. These simple reaction kinetics, if valid for other environmental conditions, are suitable for use in model systems in simplifying the analysis under conditions of constantly varying temperatures. The two thermal inactivation systems examined will be useful in the study of the thermal effects of the high temperatures, short exposure times, and constantly varying temperature resulting from laser radiation.

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<p>The kinetics of thermal inactivation of <i>E. coli</i> K12/37 and its intracellular enzyme, β-galactosidase, have been determined within a limited temperature range. Since their reaction rates are simple, these two systems can be incorporated into a system for the evaluation of a model which mathematically predicts laser injury.</p>		

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